

# Tissue Distribution of Several Series of Cationic Surfactants in Rainbow Trout (*Oncorhynchus mykiss*) Following Exposure via Water

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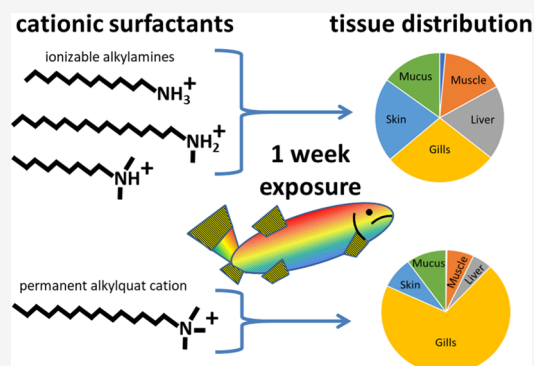


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Supporting Information

**ABSTRACT:** Bioaccumulation assessment is important for cationic surfactants in light of their use in a wide variety of consumer products and industrial processes. Because they sorb strongly to natural surfaces and to cell membranes, their bioaccumulation behavior is expected to differ from other classes of chemicals. Divided over two mixtures, we exposed rainbow trout to water containing 10 alkyl amines and 2 quaternary alkylammonium surfactants for 7 days, analyzed different fish tissues for surfactant residues, and calculated the tissues' contribution to fish body burden. Mucus, skin, gills, liver, and muscle each contributed at least 10% of body burden for the majority of the test chemicals. This indicates that both sorption to external surfaces and systemic uptake contribute to bioaccumulation. In contrast to the analogue alkylamine bases, the permanently charged quaternary ammonium compounds accumulated mostly in the gills and were nearly absent in internal tissues, indicating that systemic uptake of the charged form of cationic surfactants is very slow. Muscle–blood distribution coefficients were close to 1 for all alkyl amines, whereas liver–blood distribution coefficients ranged from 13 to 90, suggesting that the dominant considerations for sorption in liver are different from those in blood and muscle. The significant fraction of body burden on external surfaces can have consequences for bioaccumulation assessment.



## INTRODUCTION

Cationic surfactants are used in a wide variety of consumer products and industrial processes such as hair care products, fabric softeners, and hydraulic fracturing fluids.<sup>1–3</sup> Some have been detected in the mid  $\mu\text{g L}^{-1}$  range in filtered municipal wastewater treatment plant (WWTP) influent and in the low  $\mu\text{g L}^{-1}$  range in municipal WWTP effluent.<sup>4,5</sup> Concentrations in sediments downstream of WWTPs can reach  $\text{mg kg}^{-1}$  levels.<sup>6</sup> Cationic surfactants sorb very strongly to phospholipid membranes.<sup>7</sup> This affords them the ability to disrupt cell membranes in any organism, which in turn contributes to a high potential to exert baseline toxicity (narcosis).<sup>8,9</sup> Quite a number of cationic surfactants are used for their biocidal activity, as antiseptics and preservatives.<sup>2,10</sup> Some have been shown to possess genotoxicity or developmental toxicity.<sup>3</sup> Given the large volumes of cationic surfactants used in applications that can result in release to the environment and the toxicity that some of them possess, there is a need for understanding the bioaccumulation behavior of this group of chemicals. Bioaccumulation becomes particularly relevant in the context of exposure hazard evaluation such as vPvB assessment in the European chemical regulation REACH (<https://reachonline.eu/reach/en/annex-xiii.html>).

An important class of cationic surfactants is the strongly basic alkylamines, which possess hydrophobic hydrocarbon

chains that typically range between C10–18. Alkyl amines are often used in mixtures of different chain lengths. The alkyl chains can be linear or branched, and they can contain unsaturated bonds. Some alkyl amines contain multiple chains and/or additional polar functional groups near the amine moiety. At neutral pH, these amines exist mostly in the protonated cationic form. The quaternary alkylammonium compounds (QACs) are a second, structurally related, class of cationic surfactants. QACs are permanently charged cationic salts, where the charged ammonium moiety can be surrounded by four different hydrocarbon-based substituents or parts of (hetero)cyclic units such as pyridinium, morpholinium, or imidazolium. The structural variety of the hydrophobic moiety within this class is comparable to the amines.<sup>11</sup>

Bioaccumulation depends on the sorption properties of the organism, barriers limiting exchange of chemical between the organism and its surroundings, and susceptibility to biotransformation.<sup>12</sup> At environmental concentrations, cationic

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**Table 1. Time-Weighted Mean ( $n = 16$ ) Measured Aqueous Concentration in the Two Mixtures for the Tissue Distribution Tests and Median Relative Standard Deviation of Test Substance Concentration in Triplicate Water Samples**

abbreviation	name	concentration in aquarium ( $\mu\text{g L}^{-1}$ )		
		intended	measured (RSD)	% of intended
MIX 1				
P9	Nonylamine	50	46 (0.03)	92
T10	<i>N,N</i> -dimethyldodecylamine	25	21 (0.07)	82
P12	Dodecylamine	25	12 (0.04)	47
T13	<i>N,N</i> -dimethyltridecylamine	10	3.1 (0.07)	31
Q14	<i>N,N,N</i> -trimethyl-1-tetradecylammonium	2.5	1.3 (0.03)	50
P16	hexadecylamine	2.5	n.a.	n.a.
MIX 2				
T9	<i>N,N</i> -dimethylnonylamine	50	52 (0.05)	104
Q10	<i>N,N,N</i> -trimethyl-1-decylammonium	50	59 (0.01)	118
S12	<i>N</i> -methyldodecylamine	25	17 (0.04)	67
P13	tridecylamine	10	3.7 (0.07)	37
T14	<i>N,N</i> -dimethyltetradecylamine	2.5	0.49 (0.11)	20
S16	<i>N</i> -methylhexadecylamine	2.5	0.93 (0.05)	37

surfactants are dissolved as monomers, but because of the hydrophobic tail and positively charged amine or ammonium, these monomers have a strong sorption affinity for many environmental materials such as dissolved humic acids, suspended clay particles, and bacterial cell walls, as well as interfaces such as air–water and plastic–water.<sup>9,13,14</sup> This suggests that these substances could bioaccumulate by sorbing to the surface of organisms. However, they have also been shown to have a strong affinity for phospholipid bilayer membranes, with alkyl amines and QACs with a carbon chain  $> C_{12}$  having a log membrane–water distribution coefficient ( $\log D_{\text{MW}}$ )  $> 4.5$ .<sup>7</sup> Considering an average 1–2% phospholipid content in most fish species,<sup>15</sup> such high sorption affinities to cell membranes could result in substantial accumulation in the interior of aquatic organisms. Relatively high bioaccumulation factors between 100 and 1000 have already been reported for several basic pharmaceuticals in fish exposed in mesocosms receiving sewage treatment plant effluent,<sup>16</sup> while these compounds have lower  $\log D_{\text{MW}}$  values (4.3 for fluoxetine and sertraline)<sup>17</sup> than the cationic surfactants mentioned above.

There are almost no studies on the bioaccumulation of cationic surfactants in the peer-reviewed literature. Because of their unique chemical properties, there are challenges in maintaining consistent aqueous concentrations in experimental tests with biota. Near-constant aqueous concentrations ( $\pm 20\%$  of the mean) during the exposure phase are a requirement for a valid BCF experiment according to OECD 305 guidelines.<sup>18</sup> A review of early BCF studies concluded that there were no useful data for cationic surfactants.<sup>19</sup> The little information that there is on this subject comes largely from regulatory dossiers that refer to unpublished studies conducted to fulfill chemical registration requirements. Bioconcentration of  $^{14}\text{C}$ -labeled didecylmethylammonium chloride was measured in bluegill fish (*Lepomis macrochirus*), yielding a bioconcentration factor (BCF) of 81  $\text{L kg}^{-1}$ . It was reported that  $^{14}\text{C}$  residues were 2–6 times higher in the skin than in edible tissues.<sup>20</sup> A BCF of 79  $\text{L kg}^{-1}$  was reported for  $^{14}\text{C}$  alkyl( $C_{12}$ – $C_{16}$ )dimethylbenzyl ammonium chloride in bluegill fish as determined in a 35/21d uptake/depuration study, with reported measurements of edible (muscle) and nonedible parts (viscera/carcass).<sup>21</sup> The ECHA Registration dossier for octadecylamine refers to an attempted OECD 305 flow-

through BCF test with hexadecylamine and carp (*Cyprinus carpio*), indicating both technical difficulties in maintaining constant aqueous exposure concentrations and substantial adsorption of the test compound to the outer mucous layer (<https://echa.europa.eu/registration-dossier/-/registered-dossier/14418/5/4/2>). With an acidic methanol wash to remove the mucous from the fish, a BCF of 300  $\text{L/kg}$  was reported, with acknowledged uncertainty about the validity of a BCF determined in this manner. In summary, there are few peer-reviewed studies on how, in which tissues, and to what extent cationic surfactants bioaccumulate.

Given the high inherent partitioning potential and paucity of experimental bioaccumulation data for cationic surfactants, it is clear that further bioconcentration studies are needed. However, in light of the difficulties that have been reported, it must first be ascertained what tissues are relevant for fish accumulation of cationic surfactants and whether constant exposure concentrations can be sustained in a bioconcentration experiment. In this work, we undertook to measure the tissue distribution of analogue series of cationic surfactants in fish following exposure via water in a flow-through system. We chose much larger fish than would be used in a bioconcentration experiment in order to allow determination of surfactant residues in a range of fish tissues. We designed the sampling to allow us to explore the contribution of sorption on external surfaces to the total chemical burden in the fish, and whether this increased with increasing hydrophobicity of the surfactants. Blood, muscle, and liver were analyzed to quantify residues that had been taken up systemically, and mucus, skin, and gills were analyzed to quantify external residues or residues at the interface between water and internal tissues. Twelve alkyl amines and QACs were studied as model cationic surfactants, chosen to provide insight into how physical chemical properties such as chain length ( $C_9$ – $C_{16}$ ) and amine type (substitution of hydrogens on the amine by methyl groups) influence the tissue distribution.

## METHODS

**Test Chemicals and Reagents.** Four primary amines (abbreviated P), two secondary amines (S), four tertiary amines (T), and 2 QACs (Q) were studied (see Table 1 for the abbreviations used, Table S1 in the Supporting Information for structures, purity and the suppliers, and

Table S2 for properties). The length of the alkyl chain was restricted to a range that—based on existing partitioning data—would make it amenable to study in a fish bioconcentration experiment, that is sufficiently bioaccumulative but without excessive sorption issues.<sup>7,14</sup> The test chemicals were grouped into two mixtures of six, whereby each chemical in a mixture had a different alkyl chain length (Table 1). In this manner, it was ensured that possible biotransformation products formed by demethylation of the amine group were not in the same mixture. Studying chemical mixtures has the advantage that differences in behavior between chemicals are not obscured by biological variability or experimental variables.<sup>22</sup> Bioconcentration studies with mixtures have been shown to provide similar results to studies with single chemicals.<sup>23</sup>

The quality and supplier of the solvents used are listed in Table S3. Analytical standards were prepared in methanol and stored in glass. Polypropylene vials were employed for storing all extracts and solutions.

**Fish Exposure and Sampling.** The study was conducted with rainbow trout (*Oncorhynchus mykiss*) weighing between 98 and 165 g. The fish were purchased from Nordic trout Sweden AB and held in the aquaria facility prior to the experiment in February 2018. Ethical approval for the experiments was obtained from Stockholms djurförsöksetiska nämnd (decision 9967-2017). The experiments were conducted in 300 L fiberglass aquaria with a water renewal rate of 1.3 L min<sup>-1</sup> (MIX 1) and 1.45 L min<sup>-1</sup> (MIX 2). The water temperature was 10 °C and the pH 7.5. The water hardness was estimated to be 1.1 mM Ca<sup>2+</sup> based on information from the water supplier. The aquaria water was circulated through a filter of polyester wool at 800–1200 L h<sup>-1</sup> with an Eheim 2273 Pro 4+ pump. The outflow from the filter pump was placed above the water surface, providing for aeration. The lighting was dim and programmed on a 12 h light/12 h dark cycle. The fish were fed fish food pellets supplied by the fish farm at 1.0% of their body weight per day. No changes in fish behavior or appearance were observed during the experiment.

A solution of the test chemical mixture in methanol was infused continuously (3.5 and 3.8 μL min<sup>-1</sup> for MIX 1 and MIX 2, respectively) into the water inflow using a syringe pump. The intended concentrations of the chemicals in water ranged from 2.5 to 50 μg L<sup>-1</sup> (Table 1). They were selected with the help of modeled estimates of the BCF that were based on reported and extrapolated membrane water partition coefficients<sup>7</sup> and biotransformation rate constants predicted with quantitative structure activity relationships (QSARs)<sup>24,25</sup> to parameterize the BIONIC model.<sup>26</sup> Based on these estimates, we selected an exposure concentration which would result in concentrations in fish that were clearly above the limit of quantification (LOQ) of the analytical method, while trying to keep the quantifiable exposure concentration as low as possible to minimize the risk of toxic effects and micelle formation (see comparison of intended concentrations with available toxicological data in Table S4).

For each mixture, the syringe pump was started in an aquarium containing no fish. After 16 h, to allow the concentrations to stabilize, 12 rainbow trout were added. After 7 d of exposure, the fish in the exposure aquaria as well as several unexposed (control) fish were sacrificed. Following stunning, blood was collected with a heparinized syringe from the caudal vein into a 2 mL Eppendorf tube. The fish were weighed and photographed, and the length was measured. The

outer surface area of the fish was estimated from the fish weight according to O'Shea et al.<sup>27</sup>

$$(\text{surface area, in cm}^2) = 11.2 \cdot (\text{body weight, in g})^{0.65} \quad (1)$$

The gill surface area was estimated from the fish weight according to the equation specific for *O. mykiss* from a report by Hughes,<sup>28</sup> taken from ref 29

$$(\text{gill surface area, in cm}^2) = 3.15 \cdot (\text{body weight, in g})^{0.932} \quad (2)$$

The surface of the fish posterior of the gills was rinsed with 100% methanol to remove test chemical residues adsorbed to the outer surface of the skin and absorbed in the skin mucus. Methanol is an effective solvent for extracting the test chemicals from tissue, as shown below. The fish were dissected and the liver, the kidney, the gills, and the remaining contents of the abdominal cavity were taken and weighed. Skin and muscle samples were prepared from the upper dorsal region on semi-frozen fish after the methanol rinse had removed the mucus. The main part of the subcutaneous fat tissue was included in the muscle samples rather than in the skin samples. All samples were stored frozen at -20 °C until further analysis.

**Preparation of Fish Samples.** For 6 fish from each aquarium and 3 control fish, samples of muscle, skin, liver, and gills were homogenized in a bullet blender (muscle and liver) (MiniG, SPEXsamplePrep) or in a cryo-mill (skin and gill) (Mixer mill cryomill, Retsch GmbH). A sub-sample of 0.5–1.2 g of the homogenate was extracted twice in methanol (4 mL, 50 °C, ultra-sound, 60 min), employing centrifugation at 4000 rpm for phase separation. Further information on the extraction efficiency of this method can be found in the Supporting Information (Table S5 and accompanying text). Isotope labeled standards of Q10 (D<sub>21</sub>), Q14 (D<sub>29</sub>), and P16 (D<sub>33</sub>) were added to a portion of the extract corresponding to 12–75 mg of the sample. The extract volume was adjusted to 3 mL with methanol and then cleaned up on a weak cationic exchange SPE column (WCX, 60 mg, 30 μm particle size, waters). Solvent elution was performed by gravity flow. The column was conditioned with 2 mL of methanol, followed by 2 mL of Milli-Q water adjusted to pH 7 with ammonium hydroxide to activate the ion exchange. The sample extract was loaded onto the column, and it was subsequently rinsed with 20 mL of methanol. The analytes were released by first neutralizing the charged sites in the sorbent by the addition of 0.5% trifluoroacetic acid in Milli-Q water. The remaining water in the column was blown out with nitrogen. The surfactants were subsequently eluted from the WCX column with 0.65 mL of methanol. The remaining methanol was pushed out with nitrogen.

Whole blood was analyzed rather than plasma because of the small quantity of sample available and the anticipated low concentrations. Approximately 220 mg was transferred to a 13 mL polypropylene tube (Sarstedt AG & Co). After addition of internal standard, the blood was extracted with 3 mL of methanol using the same method employed for the other tissues. An aliquot of 1.5 mL of the combined extract was cleaned up on the WCX column using the same procedure employed for the other tissues, except that the column was rinsed with 10 mL (instead of 20 mL) after addition of the extract.



The methanol rinse of the fish surface (hereafter called “mucus”) was analyzed via direct injection after addition of internal standards.

**Instrumental Analysis.** For instrumental analysis, 30  $\mu\text{L}$  of purified extract was separated on an Acquity UPLC BEH C18 column ( $2.1 \times 50$  mm, particle size  $1.7 \mu\text{m}$ ) and analyzed on a Waters Xevo TQS triple quadrupole mass spectrometer using electrospray ionization. The mobile phase was a binary gradient of  $\text{H}_2\text{O}$  and methanol (mobile phase A, 95:5  $\text{H}_2\text{O}$ /methanol, v/v; mobile B, 5:95  $\text{H}_2\text{O}$ /methanol, v/v; 10 mM ammonium acetate in both). All analytes were analyzed in positive mode using multiple reaction monitoring. The separation gradient program and the optimized mass spectrometer parameters are described in the [Supporting Information](#) (Tables S6 and S7). The methanol eluate was diluted with 20–30% Milli-Q water before analysis to improve the chromatography.

Internal standard calibration was used. An 11-point calibration curve was employed with the analyte concentrations ranging from 0.06 to 130  $\text{pg } \mu\text{L}^{-1}$ . Calibration standards were prepared in 1.5 mL polypropylene vials containing water. The methanol/water ratios in the vials were 40:60 for water analysis and 80:20 for the fish analysis. The calibration was linear over the highest 8–10 standards with  $r^2$  values  $\geq 0.99$ . [Table S1](#) shows which labeled standard was used for the quantification of each analyte.

**Water Samples.** During the exposure experiments, water samples were collected. Special care was taken to minimize sample handling and in particular contact with surfaces during handling and to mix the water with solvent as quickly as possible in order to minimize sorption losses of test chemicals. Triplicate water samples were taken just before adding the fish, hourly for the first 8 h of exposure, and daily thereafter. Aquarium water (600  $\mu\text{L}$ ) was sampled with an auto-pipette (polypropylene tip). The syringe was slowly filled and emptied five times before the sample was collected to avoid losses to the pipette tip inside surface.<sup>14</sup> The sample was transferred to a 1.5 mL polypropylene sample vial containing 900  $\mu\text{L}$  of methanol and isotope labeled standards of Q10, Q14, and P16. The water/methanol mixture (60  $\mu\text{L}$ ) was then analyzed using liquid chromatography with tandem mass spectrometry as described for fish.

The possibility of sorption losses to the vial was explored by spiking standard for 8 of the test substances into 0.5 mL of different water methanol mixtures in polypropylene vials and storing the vials at 4  $^\circ\text{C}$  overnight. For water/methanol ratios of 2:3 and 1:4, the recovery of all test analytes was  $>80\%$  (see [Figure S1](#)). We note that the strong tendency of the longer chained test chemicals to sorb to surfaces precluded the use of filtration or sorption-based methods to separate out the free (dissolved) fraction.

## RESULTS

**Quality Assurance.** The repeatability of the water method, measured as the average relative standard deviation of the triplicate samples collected at each time point, ranged between 1 and 11% ([Table S8](#)). Poorer repeatability in a triplicate group was frequently associated with elevated concentrations of all of the more hydrophobic analytes in one of the triplicate samples. A plausible explanation is that a large particle of organic material (e.g., feces) had been collected with that particular sample. P16 could not be analyzed in the water

samples due to an interference introduced by erroneous addition of an incorrect internal standard.

The repeatability of the method for muscle and liver, measured as the relative standard deviation (RSD) of triplicate samples, varied from 2 to 29 and 1 to 12%, respectively ([Tables S9a,b](#)). The LOQ was determined as mean +  $10 \times$  standard deviation of the concentrations measured in the tissues from the control fish ([Tables S10–S15](#)). Because the concentrations in the exposed fish were not corrected for the concentrations in the control fish, a concentration below the LOQ in an exposed fish represents an upper limit of the concentration attributable to exposure. The concentrations in the exposed fish were well above the LOQ for most tissues. The major exceptions were Q10 and Q14, which were below the LOQ in muscle, liver, skin, and blood (Q10 only). P12 was also below the LOQ in three muscle samples. In this case, one control fish had much higher concentrations than the other three, which increased the LOQ by a factor of 7 ([Table S11](#)). This resulted in three of the six samples falling below the LOQ, although the six samples contained similar concentrations. Because of the uncertainty in the LOQ, these P12 data were included in the data analysis. The treatment of the data for Q10 and Q14 is described below.

**Concentrations in Water.** The mean measured concentrations in water ranged from 0.49  $\mu\text{g L}^{-1}$  (T14) to 59  $\mu\text{g L}^{-1}$  (Q10) ([Table 1](#)). The concentrations of P9, T9, T10, and Q10 were close to the intended concentrations, whereas the concentrations of the longer chained substances were markedly lower than the intended concentrations. The water concentrations reported here are bulk water concentrations, not freely dissolved water concentrations. In addition, a trend of decreasing concentrations over time by a maximum of 56% was observed for the longer chained substances ([Figure S2](#)). These observations can be explained by the stronger sorptive properties of the longer chained substances. Preliminary experiments suggested that the primary site of sorption in the aquarium system was the filter, and the increasing sorption over time could be related to the increasing accumulation of organic matter (e.g., fish excrement) on the filter over the course of the experiment.

**Concentrations in Fish Tissue.** The test chemical concentrations generally increased in the order muscle < blood < skin < gills < liver ([Table 2](#) for average concentrations, [Tables S10–S15](#) provide the concentrations in individual fish). Because the mass of extracted mucus was not determined, the concentrations in mucus were normalized to the estimated fish's total surface area ([eq 1](#)) excluding the head, which was not rinsed. The concentration in mucus was on average 3.9 (range 0.9–11.6) times lower than the surface area-normalized concentration in gills (from [eq 2](#)).

The variability in tissue concentration was lowest in gills and mucus (mean RSD 11 and 17%, respectively). These are the two tissues for which adsorption directly from the water could have played a dominant role in test chemical accumulation. The highest variability was observed for the liver (31%). One possible explanation for the higher variability in this tissue could be the large variability in liver weights, which ranged from 0.95 to 2.66 g. However, there was no significant correlation between the concentration in liver and liver weight or liver somatic index that was consistent across chemicals. Another possible explanation is individual differences in the binding capacity of the liver tissue. The mean concentrations in

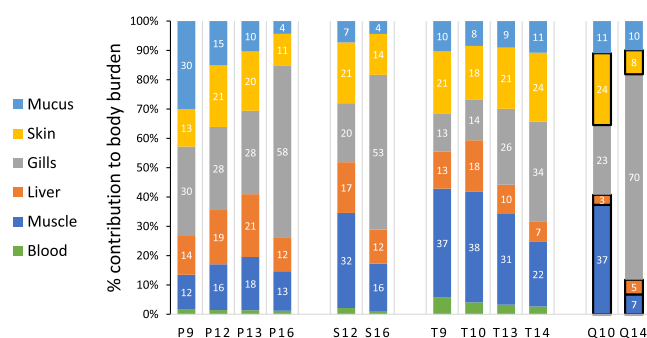
Table 2. Concentrations (ng g<sup>-1</sup> Wet Weight) of Analytes in Different Tissues Averaged Across the Six Individual Fish Sampled<sup>a</sup>

tissues ng g <sup>-1</sup>	primary alkylamines				N-methyl alkylamines			N,N-dimethyl alkylamines				N,N,N-trimethyl alkylammonium		
	P9	P12	P13	P16	S12	S16	T9	T10	T13	T14	Q10	Q14		
Blood	46 (29)	770 (26)	540 (12)	130 (19)	1090 (10)	297 (9)	330 (23)	493 (22)	930 (17)	301 (14)	1.0 <sup>b</sup> (34)	1.7 (13)		
Muscle	27 (39)	630 (28)	530 (16)	111 (22)	1230 (10)	320 (13)	160 (19)	350 (18)	670 (19)	185 (11)	3 (108)	3 (26)		
Liver	1710 (40)	42 000 (15)	50 000 (26)	5500 (10)	53 000 (30)	18 100 (24)	3900 (49)	9000 (44)	11 800 (43)	4500 (33)	15 (25)	149 (23)		
gills	1200 (12)	20 000 (10)	17 800 (12)	8700 (15)	16 400 (8)	22 000 (11)	1190 (16)	2400 (4)	10 200 (12)	6300 (13)	28 (9)	670 (16)		
Skin	132 (36)	3900 (25)	2400 (19)	420 (25)	3300 (24)	1140 (19)	390 (40)	760 (11)	2100 (17)	830 (23)	6 (87)	19 (18)		
mucus <sup>c</sup>	21 (20)	190 (12)	101 (12)	11 (13)	93 (11)	29 (14)	15 (24)	25 (18)	62 (9)	31 (13)	0.21 (19)	1.6 (26)		
water <sup>d</sup>	46	12	3.7	N.A.	17	0.93	52	21	3.1	0.49	59	1.3		

<sup>a</sup>The relative standard deviation (%) is shown in brackets. <sup>b</sup>Results in italics were below the LOQ; Q10 and Q14 in muscle, liver, and skin; Q10 in blood. <sup>c</sup>Concentrations in ng cm<sup>-2</sup>. <sup>d</sup>Concentrations in ng mL<sup>-1</sup>.

water are included in Table 2 to facilitate comparison of tissue concentrations across chemicals.

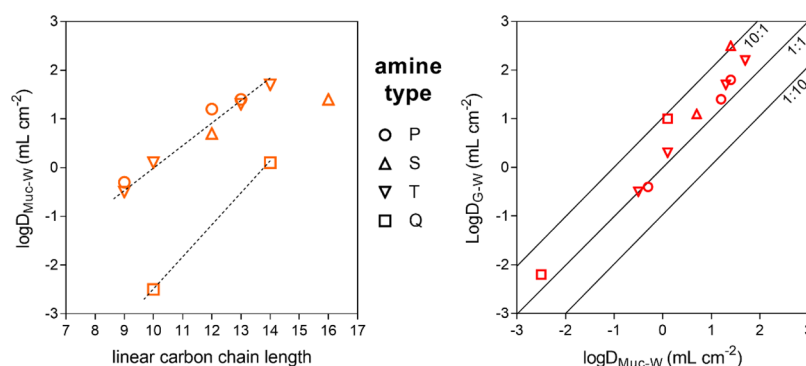
**Distribution Among Tissue Compartments.** To calculate the quantity of the test chemical in the different tissues, the amount of each tissue in the fish was estimated and multiplied by the concentration in that tissue. The test chemical quantities in the different tissues were then summed to give the body burden in each fish. The total weight of the fish and the weight of the liver and gills were available from the dissection (Table S16). For skin and mucus, the estimated outer surface area of the fish was used. We note that the distinction between mucus and skin is an operational one determined by the extraction method; mucus can be viewed as a more readily extracted fraction of the test chemical residues at the fish surface. The volume of blood was taken as 0.0495 mL g<sup>-1</sup> fish.<sup>30</sup> The remaining tissue (difference between the weight of the whole fish and the weight of liver, gills, skin, and blood) was assumed to have the concentration measured in the muscle. The contribution of the different tissues to the body burden is given for each fish in Table S17, and the average contribution across all fish is plotted in Figure 1. All of the



**Figure 1.** Mean distribution of the test chemicals among different fish tissues, expressed as contribution to the fish body burden. On the x-axis P, S, and T refer to primary, secondary, and tertiary amines, respectively, Q refers to quaternary ammonium, and the numbers refer to the length of the alkyl chain. The category muscle includes an estimate of the contribution of all tissues not specifically analyzed (see text). The results for Q10 and Q14 are estimates, as only concentrations in gill and mucus were detected above LOQ for Q10 and Q14 (and blood for Q14); the contributions of the other tissues represent maximum estimates (highlighted in the figure with a black border), as they were calculated with concentrations below the LOQ that represent upper estimates of the true concentrations in the fish (see Quality Assurance above). In the minimum scenario, the contributions of these tissues would be 0, and Q10 and Q14 would be distributed between mucus and gills only.

tissues, with the exception of blood, contribute at least 10% of the body burden for at least half of the test chemicals. There is no consistent negative correlation between the fraction of chemical associated with blood, muscle, and liver (tissues where the residue originates entirely from systemic uptake) and alkyl chain length (a measure of hydrophobicity). Thus, although adsorption to fish surfaces appears to play a significant role, its contribution to body burden cannot be simply predicted from surfactant hydrophobicity. In the following, we examine uptake in individual tissues.

**Mucus.** The presence of at least 10% of half of the test chemicals in the mucus compartment suggests that sorption of the cationic surfactants to the outer surfaces of fish can be nonnegligible. Surface area-normalized mucus–water distribu-



**Figure 2.** (A) Mucus–water distribution coefficients ( $D_{\text{Muc-W}}$ , expressed as an accumulated amount of surfactant per  $\text{cm}^2$  extracted surface divided by the exposure concentration) plotted against the linear alkyl chain length for the various cationic surfactants and (B) surface area-normalized  $D_{\text{Muc-W}}$  values plotted against surface area-normalized gill–water distribution coefficients ( $D_{\text{G-W}}$ ). The letters in the legend refer to primary (P), secondary (S), and tertiary (T) amines plus QACs (Q).

tion coefficients ( $D_{\text{Muc-W}}$ , in  $\text{mL cm}^{-2}$ ) calculated from the average concentrations in mucus and water show a strong positive relationship with alkyl chain length up to C14 for the ionizable alkylamines, with virtually no influence of the amine methylation (Figure 2A). The large range in  $D_{\text{Muc-W}}$  (2 orders of magnitude) indicates that sorption of the amines to mucus is not solely due to electrostatic attraction. In the linear alkyl chain length range of C<sub>9</sub>–C<sub>14</sub>, the increment per CH<sub>2</sub> unit is  $0.44 \pm 0.03$  (s.e.), which is higher than what was observed for sorption to soil organic matter (+0.28)<sup>31</sup> but lower than that for sorption to phospholipid membranes (+0.59).<sup>7</sup> S16 does not follow the same chain length relationship as the other ionizable amines; it lies at the same level as T14. The reason for this may be that the freely dissolved concentration was lower than the bulk concentration measured in the water samples,<sup>13,32</sup> but such bioavailability issues for the most hydrophobic cationic surfactants were not studied in further detail here. The quaternary ammonium cation (QAC) Q14 has a 2.6 log units higher  $D_{\text{Muc-W}}$  value than Q10. More remarkably, the  $D_{\text{Muc-W}}$  values for the QACs were 360 and 37 times lower, respectively, than for the tertiary amines with the corresponding alkyl chain length (Table S18). It is not clear what is causing the deviation between QACs and alkylamines. For both organic matter and phospholipids, lower affinities have been reported for QACs than analogue primary amines, in the order of 0.1–0.3 log units<sup>31</sup> and 0.92–1.23 log units,<sup>7</sup> for the respective sorbent materials. Figure 2A suggests that a model based on carbon chain length could allow for extrapolation of  $D_{\text{Muc-W}}$  to other cationic surfactants, but the different behavior of S16 and the QACs indicates limitations to the applicability domain that need further study.

Mucus solids in fish are predominantly glycoproteins.<sup>33</sup> It is expected that overall the mucus is net negatively charged at near neutral pH because of the presence of substances such as sialic acid in oligosaccharide side chains.<sup>34</sup> A cation-exchange capacity has been reported for mucus of  $\sim 0.08$  mol/g dry mucus.<sup>35</sup> Hence, positively charged solutes may be electrostatically attracted into the mucus matrix and may also engage in electrostatic and/or hydrophobic interactions with the various charged biomolecules.<sup>35,36</sup> Partitioning of cationic compounds between glycoproteins and water may therefore be partly driven by the same considerations underlying sorption to dissolved organic matter (mainly ion-exchange interactions) and phospholipids (i.e., a combination of electrostatic and hydrophobic interactions in hydrophobic pockets of the

mucus). However, because of uncertainty in the actual mucus coverage on our fish, meaningful comparisons of the mucus sorption affinity with other solid materials cannot be undertaken.

Despite the apparently relatively strong sorption capacity of the thin outer mucus layer, it generally makes a small contribution to fish body burden because of the small body mass fraction of mucus. However, mucus may make a larger contribution to whole body burden when (i) the mucus to body weight ratio is higher (e.g., smaller fish, other fish species, other aquatic organisms), (ii) the surfactant is rapidly biotransformed, or (iii) the surfactant is not significantly taken up by the fish (as is apparently the case for the QACs; see below).

**Gills.** The gills are the tissue with the largest proportion of the body burden for P16, S16, T14, and Q14 in all individual fish and for P12 and P13 in four of five individual fish (Table S17). Surface area-normalized gill–water distribution coefficients ( $D_{\text{G-W}}$ ) were calculated from the average concentrations measured in gills and water, and a calculated gill surface based on rainbow trout specific scaling factors (eq 2). Figure 2B shows that surface area-normalized distribution coefficients for gills and mucus,  $D_{\text{G-W}}$  and  $D_{\text{Muc-W}}$ , follow the same trends and are of the same order of magnitude, with gill concentrations on average a factor of 3.9 higher. As with mucus,  $D_{\text{G-W}}$  showed a strong positive relationship with alkyl chain length, but little influence of the head group for the amines. Also comparable to mucus,  $D_{\text{G-W}}$  was much lower for the QACs Q10 and Q14 (a factor of 300 and 18, respectively, Table S18) than for the tertiary amines of the same alkyl chain length. This could be related to the fact that fish gills are also coated in a mucus layer.<sup>34</sup> Unlike the skin, the gills were not rinsed with methanol, so the gill samples included gill mucus. However, because the whole gill was extracted, some portion of the chemical residues in the gill samples may have been absorbed into gill tissue. The experimental protocol did not allow us to distinguish between the chemical sorbed to mucus and the chemical absorbed into the gill.

The interior tissues (blood, liver, and muscle in Figure 1) accounted for more than half of the body burden of three of the amines (S12, T9, and T10) and more than a quarter of the body burden of the remaining amines. This indicates that gill uptake and distribution in the interior tissues are important for the bioaccumulation of alkyl amines in fish. The skin contained about 20% of the body burden of most of the amines.



Chemical residues could have reached the skin directly from water (via the mucus layer) or via the gills and internal circulation. The absence of quantifiable levels of Q10 and Q14 in any of the interior tissues (with the exception of Q14 in blood, which was just above the LOQ) suggests that the uptake of the QACs into interior tissue is very slow. In a study of the distribution of another QAC, hexadecylpyridinium bromide, in tadpoles following a 24 h exposure via water, concentrations in liver, kidney, and “fat body” were 30–100 times lower than the concentration in gills.<sup>37</sup>

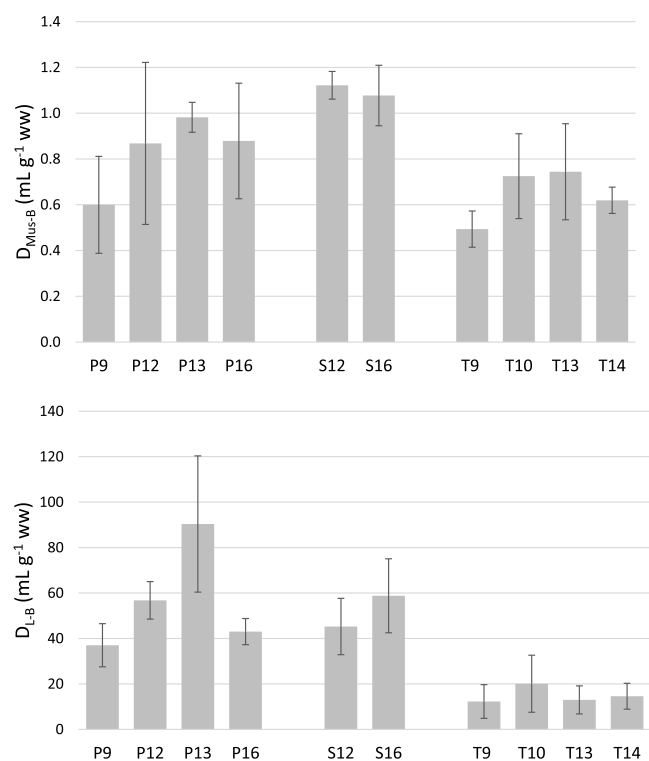
**Tissue–Blood Distribution.** Blood is the transport medium for contaminants in fish, and the tissue distribution is governed by tissue–blood distribution coefficients. Of the tissues sampled in this study, tissue–blood distribution can be unambiguously studied for muscle and liver (for gills and skin, contact with water could have influenced the residue levels). Muscle–blood and liver–blood distribution coefficients ( $D_{\text{Mus-B}}$  and  $D_{\text{L-B}}$ ) were calculated as the quotients of the concentrations in the respective tissues and blood. This approach implies that there was an internal test chemical steady state in the fish. While there may not have been a steady state between the internal tissues and the external exposure medium (see below), a near-steady state situation for chemical distribution among the major internal tissues is not an unreasonable assumption after 7 days of constant exposure.

Muscle–blood distribution was much more uniform across the test chemicals than mucus–water and gill–water distribution (Figure 3). Examining the mean values of  $D_{\text{Mus-B}}$  for the six fish, the maximum (for S12) and minimum (for T9) differ by just a factor of 2.3. There is no consistent

trend with chain length or methyl substitution of the amine group. This similarity in the  $D_{\text{Mus-B}}$  values suggests that the nature of the dominant sorbent is similar in blood and muscle, while the fact that the values are close to 1 suggests that the quantity of this dominant sorbent in the two tissues is similar. Given the sorption behavior of basic pharmaceuticals,<sup>38</sup> phospholipids are expected to be the major sorbent for cationic surfactants in muscle tissue and potentially blood. Furthermore, the phospholipid content of rainbow trout muscle (1.65%)<sup>39</sup> is similar to the lipid content of rainbow trout blood (1.4%),<sup>40</sup> whereby the majority of the lipids in fish plasma are primarily in the form of lipoproteins,<sup>40</sup> which may differ in sorptive capacity from phospholipids.

Compared to  $D_{\text{Mus-B}}$ , the mean values of  $D_{\text{L-B}}$  vary more, ranging over a factor of 7 (Figure 3). Furthermore,  $D_{\text{L-B}}$  is clearly lower for the tertiary amines than for the primary and secondary amines. This suggests that the dominant sorbent phase and/or related considerations in the liver are different from those in blood (and by extension, muscle). Furthermore, the magnitude of  $D_{\text{L-B}}$  (13–90) suggests that the quantity or the specific sorption capacity of the dominant sorptive phase in the liver is markedly higher than in blood, and the lower values for  $D_{\text{Mus-B}}$  ( $\sim 1$ ) suggest that it is also markedly higher than in the muscle. Basic pharmaceuticals such as the selected serotonin reuptake inhibitors fluoxetine and sertraline, secondary amines with a  $\text{pK}_{\text{a}} > 9.5$  like the ionizable alkylamines studied here, accumulate to greater extents in the liver than in the muscle of fish exposed to sewage treatment plant effluents,<sup>41,42</sup> but not in fish subjected to controlled exposure.<sup>43</sup> Also amitriptyline, a phospholipophilic tertiary amine-based drug ( $\text{pK}_{\text{a}}$  9.8,  $\log D_{\text{MW}}$  3.9)<sup>44</sup> showed higher accumulation in fish liver than in muscle (gilt-head bream) in a controlled bioconcentration study,<sup>45</sup> but the liver/plasma concentration ratios were not as high as the  $D_{\text{L-B}}$  observed here. Higher values of  $D_{\text{L-B}}$  compared to  $D_{\text{Mus-B}}$  have also been observed for chloroquine, a basic pharmaceutical, and attributed to greater lysosomal sequestration in liver cells,<sup>38</sup> a process that has also been demonstrated for basic psychotropic drugs in slices of various rat tissues.<sup>46</sup> However, given the similarity in dissociation constants ( $\text{pK}_{\text{a}}$ ), this phenomenon is insufficient to explain the differences in  $D_{\text{L-B}}$  with respect to the amine substitution pattern observed in this study (i.e., T vs P, S amine). Further work is required to identify the nature of the dominant sorbent(s) and related considerations in the liver and assess the potential relevance for other internal organs/tissues.

The experimental data also allow estimation of the volume of distribution ( $V_{\text{d}}$ ), an important parameter for extrapolating in vitro measures of e.g. metabolism to in vivo. In the context of quantitative in vitro–in vivo extrapolation for fish,  $V_{\text{d}}$  is defined as the quotient of the concentration in fish and the concentration in blood and describes the equivalent volume of blood that would contain the same amount of the chemical as 1 kg of fish.<sup>40</sup>  $V_{\text{d}}$  was estimated using the residues that had been clearly taken up systemically (i.e., those in muscle, liver and blood). The mean  $V_{\text{d}}$  ranged from 0.49 L  $\text{kg}^{-1}$  for T9 to 1.49 L  $\text{kg}^{-1}$  for P13 (Table S19). This compares with plasma concentration based  $V_{\text{d}}$  values of other weak bases: 3.0 L  $\text{kg}^{-1}$  for diphenhydramine in fathead minnows<sup>47</sup> and 0.32–0.48 and 0.19–0.28 L  $\text{kg}^{-1}$  for diphenhydramine and diltiazem, respectively, in killifish.<sup>48</sup> These comparatively low values are an indication that a steady state for chemical distribution



**Figure 3.** Muscle–blood (upper panel) and liver–blood (lower panel) distribution coefficients. The mean and standard deviation for the 6 fish are shown. On the x-axis, P, S, and T refer to primary, secondary, and tertiary amines, respectively, while the numbers refer to the length of the alkyl chain.

among internal tissues should be approached quickly.  $V_d$  was similar in magnitude to  $D_{Mus-B}$ .

**Consequences for Bioaccumulation of Cationic Surfactants.** Apparent BCFs ( $BCF_{app}$ ) at the end of the 7-day exposure were calculated by dividing the surfactant body burden (including mucus) by the fish mass, and dividing this by the average measured concentration in water samples taken during the exposure phase. The  $BCF_{app}$  values ranged from 0.1 to 1260 (Table S18). Given the short exposure period, it is possible that the fish in this study had not approached steady state, in particular with respect to the distribution between water and internal tissues and between water and skin, and that the steady-state BCFs are higher. The proximity of some of the  $BCF_{app}$  values to the regulatory threshold for PBT chemicals in REACH (a BCF of 2000) indicates that further study of the bioaccumulative properties of cationic surfactants is warranted, whereby the longer chained alkyl amines appear to be the most bioaccumulative. The successful recoveries of a wide range of cationic surfactants from both water and fish tissues samples demonstrate that bioconcentration studies with fish with cationic surfactants are feasible. We note that some improvements in the methodology would be helpful, particularly with respect to the stability of the aqueous exposure concentration of the longest chain surfactants. Working with mixtures of analogue cationic surfactants allowed for consistent evaluation of differences in accumulation trends due to structural features.

The tissue distribution results raise a number of questions regarding the assessment of bioaccumulation of cationic surfactants. If we presume that much of the test chemical in the gills did not enter the internal circulation system, then a significant fraction of the test chemical present in the whole fish was not able to reach other target tissues and thus was constrained in its ability to exert adverse effects on the fish. This is most apparent for Q14, P16, and S16, for which at least 50% of the body burden was present in gills and mucus. Similarly, much of the chemical residue in the fish was not present in tissues that would normally be subject to human consumption [e.g., Q10 and Q14 were not present in muscle above the LOQ, and for the primary amines the category muscle (which included other tissues) contributed at most 18% to the body burden]. Consequently, humans eating just the muscle of a fish would be exposed to much lower levels of these chemicals than predators eating the same fish. This suggests that edible tissue analysis could be more appropriate than whole fish analysis for human exposure assessment. Whole fish analysis would be relevant from the perspective of biomagnification and ecological exposure assessment or if adverse effects result from adsorption to epithelial tissues (e.g., the gills). To be relevant for biomagnification, there must be efficient dietary uptake of the chemical. Still, predicted steady-state BCFs based on experimental phospholipid–water distribution coefficients suggest that QACs should accumulate to a similar extent as ionizable analogue amines. Prolonged exposure studies with smaller sized fish, including adequate uptake and elimination phases, are the logical next step to better assess whether bioconcentration of permanently charged surfactants is mainly limited kinetically and to further improve parameterization of models to predict BCF and toxicokinetics for permanently charged compounds.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.9b07600>.

Chemicals, test chemical properties, solvents, comparison of intended concentrations in water with LC50 values, extraction efficiency, liquid chromatography program, MS/MS MRM parameters, repeatability of water analysis, repeatability of fish muscle and liver analysis, concentrations of test chemicals in blood, muscle, liver, gills, skin, and mucus, mass of the fish and tissues sampled, contribution of each tissue to the fish's body burden of the test chemical, mucus-water and gill-water distribution coefficients, bioconcentration factors, estimated volume of distribution, test chemical recovery from vials made of different materials, and concentration of test chemicals in water during the fish exposure (PDF)

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### Notes

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